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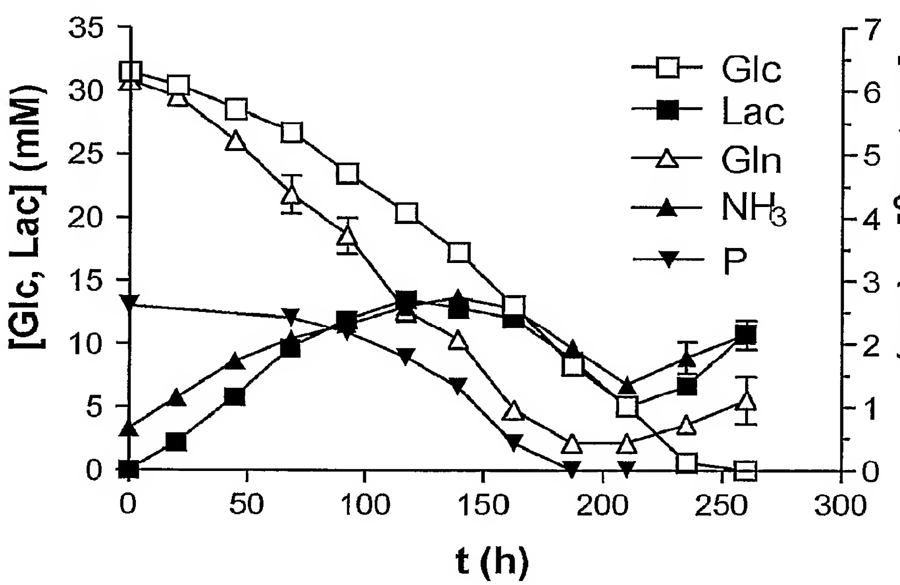
(54) **Title:** CULTURES OF E1-IMMORTALIZED CELLS AND PROCESSES FOR CULTURING THE SAME TO INCREASE PRODUCT YIELDS THEREFROM



(57) **Abstract:** The invention provides processes for culturing cells derived from embryonic retinoblast cells immortalized by adenovirus E1 sequences, preferably PER.C6™ cells, to improve product yields from such cells. Feed strategies for such cells and cultures with very high cell densities are provided, resulting in high yields of products, such as recombinant antibodies.

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TITLE OF THE INVENTION

Cultures of E1-immortalized cells and processes for culturing the same to increase product yields therefrom.

FIELD OF THE INVENTION

The invention relates to the field of cell culture. In particular, the invention relates to the field of culturing cells derived from cells that have been immortalized with E1 sequences from adenovirus. More in particular, the invention relates to culturing such cells to obtain high levels of products from such cells.

BACKGROUND OF THE INVENTION

A human PER.C6® cell line, exemplified by cells deposited at the ECACC under no. 96022940, derived from retina cells by immortalisation with the adenovirus (Ad5) E1a and E1b genes is disclosed in US patent 5,994,128. Besides the ability to function as packaging cells for E1-deleted adenoviral vectors (US patent 5,994,128; WO 01/005945), and for producing other viruses (WO 01/38362), E1-immortalized cells, such as PER.C6 cells, can be used to produce recombinant proteins, such as antibodies (WO 00/63403).

Xie et al (2002) have disclosed a process for serum-free suspension cultivation of E1-immortalized cells. However, the product yields obtained using the culturing processes disclosed in the art for E1-immortalized cells, can be improved. It is an object of the present invention to provide novel processes to increase the product yield from this type of cells.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Graph showing growth and antibody production of a PER.C6 clone (clone 1) grown in shake flask at two different starting cell concentrations (0.3 and 1.0 x 10^6 ml $^{-1}$). Left vertical axis: viable cell number (N_v). Right vertical axis: antibody (Ab) concentration. Horizontal axis: time (hours).

Fig. 2. Graph showing the decrease in the cell specific utilisation of glutamine with increasing viable cell numbers for an antibody-producing PER.C6 clone (clone 1). N: cell number.

Fig. 3. Profiles for a batch culture of PER.C6 clone 1. A: Metabolites. Glc, glucose; Lac, lactate; Gln, glutamine; NH_3, ammonia; P, phosphate. B-E: Amino acids (AA).

Fig. 4. Graph showing the effect of a feed component mix containing glucose, glutamine, amino acids, phosphate, calcium and growth factors on PER.C6 clone 1. A: viable cell numbers (N_V). B: antibody (Ab) concentration. Circles: batch. Squares: fed-batch.

Fig. 5. Graph showing viable cell numbers (N_v) and antibody (Ab) yields of PER.C6 clones where culture medium was completely exchanged once per day (2 independent experiments each). A: clone 1. B: clone 2.

Fig. 6. Result of modified feed (example 4) for clone 1. A: viable cell numbers (N_V). B: antibody (Ab) concentration. Open circles: batch. Closed circles: fed-batch.

Fig. 7. Result of further improved modified feed with different first and subsequent feed additions (example 4,

Table 2) for clone 1. A: viable cell numbers (N_v). B: antibody (Ab) concentration. Circles: batch. Squares: fed-batch. Arrows: last feed.

Fig. 8. Result of further improved modified feed with different first and subsequent feed additions (example 4, Table 2) for clone 2. N_V: viable cell number. Ab: antibody concentration.

Fig. 9. Galactosylation levels of IgG produced according to processes according to invention.

Fig. 10. Result of further improved modified feed with different first and subsequent feed additions (example 4, Table 2) for clone 3. N_V: viable cell numbers. Ab: antibody concentration.

SUMMARY OF THE INVENTION

In one aspect the invention provides feed strategies for fed-batch or fed-perfusion cultures of cells immortalized by adenovirus E1 sequences. In one embodiment thereof the invention provides a method for the culturing of such cells, said cells capable of growing in suspension, comprising the steps of: determining at least once during the culturing of the cells the concentration of at least one medium component selected from the group consisting of glucose, glutamine phosphate, leucine, serine, isoleucine, arginine, methionine, cystine, valine, lysine, threonine and glycine, adding components to the medium during the culturing of the cells at or prior to the depletion of at least one of the components of which the concentration was determined in the previous step,

wherein the components added at least comprise glucose, glutamine, phosphate, leucine, serine, isoleucine, arginine, methionine and cystine. Other components that beneficially may be added according to the invention, amounts and time of addition of the components are provided herein below, as well as in the claims.

It is another aspect of the invention to provide a culture of cells derived from cells immortalized by adenovirus E1 sequences, characterized in that said culture comprises at least 10 x 10^6 cells/ml. Preferably, said culture comprises at least 12 x 10^6 cells/ml, more preferably at least 15 x 10^6 cells/ml. In certain preferred embodiments the culture according to the invention comprises more than 20 x 10^6, 25 x 10^6, 30 x 10^6 or 40 x 10^6 cells/ml. Methods to obtain such cultures are also provided herein.

In yet another aspect, a method to increase cell densities and product yields from a culture of cells immortalized by adenovirus E1 sequences is provided. In one embodiment hereof, a process for culturing such cells is provided, characterized in that said process comprises a step of subculturing said cells at a seeding concentration of between 0.8 x 10^6 and 2.0 x 10^6 viable cells/ml, preferably between 0.9 x 10^6 and 1.5 x 10^6 viable cells/ml.

Preferably, the cells used in the methods of the invention are derived from retina cells, more preferably from human embryonic retina (HER) cells, such the cells deposited under ECACC no. 96022940. In certain embodiments, said cells are PER.C6 cells.

In certain embodiments, said cells can produce recombinant proteins, preferably antibodies, at high yields. In other embodiments said cells comprise recombinant adenoviral vectors having a deletion in the E1-region, or other viruses, which

can be produced on said cells in high yields using the process according to the invention. In preferred embodiments, the cells are cultured at least part of the time in a serum-free medium.

DETAILED DESCRIPTION OF THE INVENTION

The productivity of any cell line is mainly defined by three basic parameters, the specific productivity of the cell line, the peak viable cell concentration that is attainable and the length of the production process that is possible. Increases in either of these variables will lead to increases in the final product concentration and is dependent to a large extent on the cell line. In a straight batch culture, cell lines such as CHO and SP2/0 can achieve cell densities up to 4 x 10^6/ml. In fed-batch or perfusion processes the viable cell concentration is increased, and typically hybridoma cells such as SP2/0 can be cultured up to 10 x 10^6 cells/ml, while CHO can be cultured up to 6-10 x 10^6 cells/ml. The invention describes methods to increase the viable cell density of cultures of cells immortalized by adenovirus E1 sequences, preferably derived from embryonic retina cells, to attain cell densities beyond those reported in the prior art. Furthermore, the methods according to the invention can be used to obtain higher product yields from cultures of cells according to the invention.

The present invention discloses improvements in how E1-immortalized cells, such as PER.C6 cells, can advantageously be used for the production of high yields of monoclonal antibodies. It is disclosed that these cells be cultured to very high viable cell concentrations in a straight batch process (up to 14 x 10^6 viable cells/ml).

Furthermore, E1-immortalized cells, such as PER.C6 cells, are well suited to a fed-batch process as a culture of these cells unexpectedly consumes lactate and ammonia and maintains viability for long periods of time under nutrient limiting conditions. Methods to increase product yields from said cells by a feed strategy in cultures are provided herein.

With the term 'feed strategy' as used herein is meant the addition of certain identified components including but not limited to nutrients, such as sugars, amino acids, and the like, to the culture medium. The identified components are preferably added in certain amounts and at certain times, when they are required to improve product yields from the cells, such as provided herein.

E1-immortalized cells, such as PER.C6 cells, are also well suited to a perfusion process as they can be maintained at very high viable cell concentrations (up to 50 x 10^6 cells/ml with a viability of at least 85%) for long periods of time and with good final product concentrations.

Culture media

The processes of the invention generally increase the product yields from the cells compared to yields obtained with processes described in the art for the cells according to the invention. Preferably, serum-free culture media are used at least part of the time in the processes according to the invention. Preferably, the medium contains only recombinantly produced proteins, which are not of animal origin. Such culture media are commercially available from various sources. In one embodiment of the invention, VPRO culture medium (JRH Biosciences) is used for the fed-batch or (fed-)perfusion process.

Products

The methods of the invention are preferably used to produce products in cells of the invention. The processes of the present invention can be used for the improved production of antibodies, as well as other proteins (WO 00/63403). For the production of proteins, the cells of the invention suitably comprise nucleic acid encoding said proteins, in operable association with elements capable of driving expression of said proteins. Furthermore, the processes can be used for improvement of the production of recombinant adenoviral vectors having a deletion in the E1-region, in which case the cells are used as complementing cells, which in itself is known to the skilled person according to established methodology (e.g. US patent 5,994,128; WO 01/005945). Moreover, the processes according to the invention can be used to improve a process for propagation of other (non-adenovirus) viruses in the cells (WO 01/38362). Hence, products according to the invention can be recombinant proteins, such as antibodies, erythropoietin, and the like, as well as recombinant adenoviral vectors with a deletion in the E1 region, or other viruses.

Cells

The cells according to the invention are cells that have been immortalized with E1 sequences from an adenovirus, which cells are also referred to herein as E1-immortalized cells. Such cells express at least a functional part of the E1A region of an adenovirus, and preferably also at least a functional part of the E1B region. E1A protein has transforming activity, while E1B protein has anti-apoptotic activities. The cells according to the invention may be derived from any cell, including lung cells, kidney cells,

amniocytes, but preferably are derived from retina cells. They may be derived from embryonic retina cells. Preferably the cells according to the invention are human cells. A method for immortalization of embryonic retina cells has been described in the art (US patent 5,994,128). Accordingly, a retina cell that has been immortalized with E1 sequences from adenovirus can be obtained by that method. In certain preferred embodiments, the cells of the invention are derived from E1-immortalized HER cells, such as PER.C6 cells. PER.C6 cells for the purpose of the present application shall mean cells from an upstream or downstream passage or a descendent of an upstream or downstream passage of cells as deposited under ECACC no. 96022940. In addition, also the E2A region with a ts125 mutation may be present (see e.g. US patent 6,395,519) in said cell. A cell derived from a PER.C6 cell can be a PER.C6 cell infected with recombinant adenovirus or other virus, and can also be a PER.C6 cell into which recombinant nucleic acid has been introduced, e.g. comprising an expression cassette wherein nucleic acid encoding a protein of interest is operably linked to sequences capable of driving expression thereof, such as a promoter and polyA signal, wherein preferably said cells are from a stable clone that can be selected according to standard procedures known to the person skilled in the art. A culture of such a clone is capable of producing a protein encoded by said recombinant nucleic acid.

Components for feed strategies

In one aspect, the invention provides processes for culturing cells according to the invention, wherein by feed strategies according to the invention certain amino acids are added during the culturing process to replenish amino acids of

which the concentration has become or will become limiting for an optimal process and product yields. By amino acid is intended all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their derivatives. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteines to form cystine. Further, amino acid derivatives may include esters, salts, such as chlorides, sulphates, and the like, as well as hydrates. It will be understood by the person skilled in the art, that where a specific amino acid is mentioned herein, a derivative may also be used and is meant to be included within the scope of the invention. Other components such as sugars, growth factors, vitamins, etc may also be added to improve the processes according to the invention.

Feed strategies

In one aspect, the invention provides a method for producing a product in cells immortalized by adenovirus E1 sequences, in a culture medium, wherein said product is chosen from the group consisting of a recombinant protein, a virus, and a recombinant adenovirus with a deletion in the E1 region, characterized in that said method comprises a step wherein at least Leucine, Serine, Isoleucine, Arginine, Methionine and Cystine are added to the culture medium. In one aspect the invention provides a method for the culturing of cells immortalized by adenovirus E1 sequences, said cells capable of growing in suspension, comprising the steps of: determining at least once during the culturing of the cells the concentration of at least one medium component selected from the group consisting of glucose, glutamine, phosphate, leucine, serine,

isoleucine, arginine, methionine, cystine, valine, lysine, threonine and glycine, adding components to the medium during the culturing of the cells at or prior to the depletion of at least one of the components of which the concentration was determined in the previous step, wherein the components added at least comprise glucose, glutamine, phosphate, leucine, serine, isoleucine, arginine, methionine and cystine. "Depletion" as used herein is defined as the time a component has a concentration of 30% or less of the starting concentration in the culture medium. In these aspects, the determination of the concentration of at least one medium component selected from the group consisting of glucose, glutamine, phosphate, leucine, serine, isoleucine, arginine, methionine or cystine is preferred over the determination of only components selected from the group consisting of valine, lysine, threonine and glycine. In certain embodiments, the concentration of at least two medium components according to the invention is determined in the first step. In certain embodiments, the components that are added further comprise one or more of valine, lysine, threonine, glycine, asparagine, tyrosine, histidine, phenylalanine, tryptophane, calcium, LongR3 IGF-1, Long EGF and insulin. In specific embodiments, the components are added in an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of 6.0 for glucose, 2.60 in the first feed and 1.75 in subsequent feeds for glutamine, 0.70 for phosphate, 0.66 for leucine, 1.10 in the first feed and 0.55 in subsequent feeds for serine, 0.50 for isoleucine, 0.46 for arginine, 0.23 for methionine, and 0.25 for cystine. In further embodiments, the following components are further added to an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of 0.45 for valine, 0.44 for lysine, and 0.30 for threonine. In

further embodiments, the following components are further added to an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of 0.10 for asparagine, 0.13 for tyrosine, 0.10 for histidine, 0.02 for phenylalanine, and 0.06 for tryptophan. Furthermore, calcium may be added in an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of 0.02. Growth factors such as IGF, EGF, and insulin or their derivatives may also suitable be present in the growth medium. The amounts for the addition of components above may have an error margin per component of 33% or less, preferably 20% or less, more preferably 10% or less, even more preferably 5% or less. The amounts are presented per 10 x 10^6 cells/ml, and are linearly dependent on the number of cells/ml. In preferred embodiments, said components are added at between 48 hours and the moment of depletion of at least one of the medium components the concentration of which was determined in the previous step. In certain embodiments, said addition is at a time between 24 hours and just prior to depletion. In certain aspects, the invention provides a method according to the invention, wherein said cells express a recombinant immunoglobulin that is secreted into the culture medium to a level of at least 500 mg per liter, preferably at least 700 mg/l, more preferably at least 850 mg/l, even more preferably at least 1000 mg/l, still more preferably at least 1250 mg/l, still more preferably at least 1500 mg/l, still more preferably at least 1750 mg/l and still more preferably at least 2000 mg/l. In general, the addition of medium components according to the invention, i.e. in for instance a fed-batch process, results in an increase in the yield of produced product of at least 1.5x, preferably at least 2x, more preferably at least 2.5x and still more preferably about

3x or even higher, compared to the process wherein no components are added, i.e. the batch process.

In addition to use in a fed-batch process, the feed strategies of the invention can also be beneficially used in an optimized batch process, as set out in example 5.

Perfusion

Alternatively, in another aspect of the invention the entire culture medium may be exchanged. It is shown that unexpected high viable cell densities can be attained when this is applied to cells derived from retina cells immortalized by adenovirus E1 sequences. Exchanging culture medium may be performed by any means known to the person skilled in the art, including but not limited to collection of the cells by centrifugation, filtration, and the like, followed by re-suspension of the cells into fresh culture medium. Alternatively, a perfusion system may be used, wherein culture medium is either continuously or intermittently exchanged using a cell separation device such as a centritech centrifuge or passage through a hollow fibre cartridge, and the like. It is therefore another aspect of the invention to provide a process for culturing cells derived from embryonic retina cells immortalized by adenovirus E1 sequences, characterized in that culture medium is exchanged at a rate of 0.2-3, preferably 0.5-3, culture volumes per day (24 hours). Cultures obtained using this method preferably have viable cell densities higher than 20 x 10^6 cells/ml, more preferably higher than 30 x 10^6 cells/ml. In certain aspects, such cultures have cell densities higher than 40 x 10^6 cells/ml. In certain aspects such cultures are used to produce recombinant antibodies with a yield of at least 150 mg/l/day, preferably at least 200 mg/l/day, more preferably at least 300, 400, or

500 mg/l/day. Of course, also other products according to the invention can be produced by such methods. It is shown in here that one complete volume exchange of culture medium each day supports at least 30 x 10^6 viable cells/ml with antibody yields of more than 500 mg/L/day (up to 750 mg/l/day) (Fig. 5). One complete medium exchange per day corresponds to a continuous perfusion rate of 3 volumes per day, meaning that a continuous perfusion system could yield approximately at least 150-200 mg/L/day. One method to reduce this perfusion rate and thus increase antibody yields (by reducing the volume in which the antibody is secreted) is to supplement the fresh culture medium with the essential components (known as fed-perfusion). These components for antibody-producing E1-immortalized cell, such as PER.C6 cell, clones are identified herein (see example 2) and therefore it is another aspect of the present invention to provide such a fed-perfusion system, wherein the feed strategies according to the present invention are employed. A common drawback of fed-perfusion processes is the build-up of toxic metabolic by-products (such as lactate and ammonia), which can result in low cell viabilities and product yields. There is often a requirement at high cell concentrations for a high perfusion rate to remove these by-products. One advantage demonstrated for E1-immortalized cell, such as PER.C6 cell, clones according to the invention is that they are capable of utilising lactate and ammonia such that concentrations do not become problematical (see Fig. 3A). It is therefore possible to obtain an antibody yield of at least 500 mg/l/day by changing the culture medium once or twice a day. Alternatively this can be achieved by using a continuous perfusion rate of for instance 1 volume per day in combination with supplementation of the medium with a feed concentrate (fed-

perfusion). This can advantageously be combined with a cell bleed (removing a certain percentage of the cells population).

Cultures with high cell densities are advantageous for obtaining high product yields. It is therefore another aspect of the invention to provide a culture of cells derived from cells immortalized by adenovirus E1 sequences, said culture comprising at least 10 x 10^6 cells/ml. The viability in the culture is at least 80%. Preferably, the viability is at least 90%, more preferably at least 95%. The cultures according to the invention are preferably suspension cultures, meaning that the cells in said cultures are in suspension in the culture medium, such as in shake flasks, roller bottles, bioreactors, including stirred tanks, air lift reactors, and the like. The strategies disclosed herein may however also be used for cultures of cells in hollow fiber reactors, such as described by Tanase et al (1997), and for adherent cultures, such as cells on microcarriers. In one embodiment, said culture comprises at least 12 x 10^6 cells/ml. It is disclosed herein that up to 14 x 10^6 cells/ml can be obtained by a straight batch culture.

It is further demonstrated that, using medium perfusion, even higher cell densities can be achieved, up to 50 x 10^6 cells/ml. The prior art does not provide any indication that such unexpected high cell densities are obtainable. In other preferred embodiments therefore, the invention provides a culture of cells derived from E1-immortalized cells, preferably derived from retina cells, said culture comprising at least 15 x 10^6 cells/ml, preferably at least 20 x 10^6 cells/ml, more preferably at least 25 x 10^6 cells/ml. In specific embodiments, said culture comprises at least 30 x 10^6 cells/ml, or even at least 40 x 10^6 cells/ml. Cultures with at least 15 x 10^6 cells/ml according to the invention appear

obtainable by a perfusion process, meaning that culture medium is exchanged during the culturing process. The cultures according to the invention have a viability of at least 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%. Said cultures are suspension cultures. Said cultures further comprise growth medium. Said growth medium preferably is serum-free. The cells of the culture may comprise recombinant nucleic acid molecules encoding immunoglobulins, or parts or derivatives thereof, in expressible format. Such cells are capable of producing immunoglobulins in high yields. In particular, it is shown herein that a culture of cells according to the invention, wherein the medium is exchanged every day, and wherein more than 30×10^6 cells/ml are present, can provide recombinant antibody yields of at least 500 mg/l/day. The cells in said culture preferably produce at least 10 pg protein/cell/day.

The processes of the invention, especially those for recombinant protein production, can also be combined with other measures described in the art that in some cases improve product yields. Therefore, in certain embodiments of the invention the culture medium is subjected to a temperature shift before or during the production phase, e.g. by running the process at a lower temperature, e.g. between 30°C and 35°C, in the production phase (see e.g. US patent 6,506,598, and literature cited therein, which describes effects of lowering the cell culture temperature on several parameters for recombinant protein production), or by the addition of cold culture medium to the culture (wherein cold is meant to be lower than the temperature the cells are cultured in, preferably the cold culture medium having a temperature between 2°C and 8°C) when the cells are subcultured or later

during the culture process. In other embodiments, specific growth factors may be added to improve the processes according to the invention with regard to product yields. In yet other embodiments for the production of proteins, the processes according to the invention may be improved by the addition of alkanoic acids or salts thereof, such as sodium butyrate, either during the whole culture phase or only during the production phase (see e.g. US 6,413,746, and references therein, which describes effects of addition of butyrate on production of proteins in cell culture). In yet other embodiments for the production of proteins, the culture medium is subjected to a temperature or pH shift (Weidemann et al 1994, Sauer et al 2000).

It will be clear to the person skilled in the art that several aspects and/or embodiments according to the invention can be combined to provide a process for culturing cells which leads to particularly good product yields. As a non-limiting example, it is for instance possible to seed a culture of E1-immortalized cells at about 0.8×10^6 to 2.0×10^6 cells/ml, and use a feed strategy and/or exchange the growth medium during the culturing process to improve the final product yields.

The invention will now be illustrated with some examples, not intended to limit the scope of the invention.

Experimental

Methods and vectors for genetically engineering cells and/or cell lines to express a protein of interest are well known to those of skill in the art; for example, various techniques are illustrated in Current Protocols in Molecular Biology, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly

updates) and Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Laboratory Press, 1989). General and standard cell culture techniques are known to the person skilled in the art, and are for instance described in R.I. Freshney, Culture of animal cells: A manual of basic technique, fourth edition (Wiley-Liss Inc., 2000, ISBN 0-471-34889-9). Such standard techniques were followed unless otherwise noticed.

Cell Culture Protocols

PER.C6 cells were cultured in the examples. Cells were adapted from adherent cultures in DMEM containing 10% FBS (Invitrogen) to serum free medium by direct transfer. Briefly, sub-confluent, logarithmic cells were trypsinised, washed once with serum free medium and inoculated directly into 250 ml Ehrlenmeyer flasks with a 0.2µ filter (Corning), containing 25 ml of ExCell-525 serum-free medium (JRH Biosciences) at a starting cell concentration of 0.3-0.5 x 10^6 ml^{-1}, unless otherwise noted. Cultures were maintained in logarithmic growth in Ehrlenmeyer flasks by passage every 2-3 days. Flasks were shaken on a magnetic shaker platform (Infors) at 100 rpm in a humidified incubator at 37°C and 5% CO_2. Cultures were passaged by centrifugation at 1000 rpm for 5 minutes. The supernatant was removed and the pellet re-suspended in the remaining medium. Fresh, cold medium (4°C) was added and new flasks inoculated at the appropriate cell concentration. After transfer to serum-free medium, cultures were passaged for 2-4 weeks to allow for complete adaptation, after which a serum-free cell bank was created. All experiments were started using cells from this cell bank.

Bioreactors

Bioreactor cultures were performed in 3L reactors with a 2L working volume (Applikon). Temperature was maintained at 37°C by a heating blanket. Dissolved oxygen concentration (dO_2) was controlled at 50% of air saturation by adjusting inlet gas composition through the headspace and intermittent sparging through a microporous sparger. Starting culture pH was controlled at 7.3 by CO_2 addition through the microporous sparger. The lower culture pH limit was set at 6.7 so that the culture pH was allowed to drift downwards (the lower limit was not reached). Cultures were agitated by two marine impellers at 75 rpm. Process data was acquired by the BioExpert software (Applikon).

Analytical Protocols

Cell counts and viability measurements were performed using a CASY automatic cell counter (Schärfe Systems). Glucose, lactate, ammonia and phosphate concentrations were determined using an Ektachem II analyser (Kodak) with cell-free culture supernatants. Amino acid concentrations were determined using a modified AccuTag HPLC method (Waters) as described by van Wandelen and Cohen (1997). Aliquots (200 µl) of centrifuged culture supernatant were stored at -20°C in 1 ml cryovials (Nalgene) until required. Samples from each experiment were analyzed at the same time to avoid experimental variation. Osmolality was measured by a freezing point depression osmometer (Osmomat 030-d, Gonotec). Antibody concentration was determined by a sandwich-type ELISA. Biefly, plates were coated with 2 µg ml^{-1} mouse anti-human IgG against the kappa light chain (Pharmingen) and incubated overnight at 4 °C. An HRP-conjugated mouse anti-human IgG against the heavy chain (Pharmingen; 1:500) was used as detection antibody for 1 hr at

37 °C with OPD (Sigma) as substrate. Washing between incubation steps was performed with 0.05 % Tween 20 in PBS. Samples were diluted in washing buffer supplemented with 0.1 % BSA. Quantification was relative to an IgG1 reference standard using a calibration range of 10 to 400 ng ml^{-1}. Antibody samples purified by Protein A were subject to quality analysis by isoelectric focusing (IEF) and denaturing polyacrylamide gel electrophoresis (SDS-PAGE). For glycan analysis, N-linked glycans were removed by PNGase F treatment of the IgG samples in 20 mM sodium phosphate (pH 7.2) and analyzed with MALDI-MS in the reflector mode on an Applied Biosystems Voyager DE Pro mass spectrometer. The matrix was 2,5-dihydroxybenzoic acid (10 mg ml^{-1}) in 50/50/0.1 acetonitrile/water/trifluoroacetic acid. Spectra were obtained in the positive ion mode and glycans were detected as sodium adducts, [M+Na]+.

<u>Calculation of Cell Specific Metabolic Rates</u>

Cell specific rates of metabolite utilisation and production in batch and fed-batch culture were calculated using the log mean of the cell concentration as shown in the following equation:

$q_s = (C_2 - C_1) / (t_2 - t_1) \times [(X_2 - X_1) / \ln(X_2 - X_1)]$.

In this equation, C is the metabolite concentration (µmoles/l), t is time (days) and X is the viable cell concentration. A rate constant accounting for the spontaneous decomposition of glutamine was not included as decomposition was not significant at the time points at which the rates were calculated (data not shown). The yield coefficients of lactate produced per glucose ($Y_{lac/glc}$), ammonia produced per glutamine ($Y_{amm/gln}$) and alanine produced per glutamine ($Y_{ala/gln}$) were calculated from the equations below and are expressed in mole/mole:

$Y_{lac/glc} = q_{lac} / q_{glc}$

$Y_{amm/gln} = q_{amm} / q_{gln}$

$Y_{ala/gln} = q_{ala} / q_{gln}$

EXAMPLES

Example 1: Increasing maximum final cell yields in batch culture of PER.C6 cells

The simplest production process is a batch culture. However, this is restricted in the viable cell concentration and therefore the product yields attainable, due largely to nutrient limitation. A method is presented to increase the maximum final cell concentration of a batch culture of PER.C6 or PER.C6 derived sub-clones by calculating the cell specific rate of utilisation of key nutrients at different cell concentrations and starting the batch culture at a cell concentration where there is optimal utilization of nutrients with respect to cell growth.

The DNA encoding the antigen-binding region of an antibody recognizing epithelial cell adhesion molecule (EpCAM) was first isolated from a scFv phage display library (Huls et al, 1999). DNA encoding the antigen-binding region of an antibody recognizing CD46 was isolated as disclosed in WO 02/018948. A leader sequence and constant regions of IgG1 type were added essentially as described in Boel et al, 2000. The DNA encoding the light and heavy chains were then cloned into expression vector pcDNA3002(Neo). The expression vector pcDNA3002(Neo), which has been described in international patent application PCT/NL02/00841, was deposited on December 13, 2001 at the European Collection of Cell Cultures (ECACC) under number 01121318. The resulting expression vectors, encoding an IgG1 that recognizes EpCAM or CD46, respectively,

regulated by a CMV promoter, was introduced in PER.C6 cells according to standard methods.

A recombinant antibody-expressing clone, derived from a parental population of the PER.C6 cell line, was used in these experiments. The clone expressing anti-EpCAM is further referred to herein as clone 1, the clone expressing anti-CD46 is further referred to herein as clone 2.

Cells were maintained in ExCell™ 525 medium (JRH Biosciences) (maintenance of the cells in GTM-3 medium (Sigma) did also work) and batch productions were carried out in ExCell™ VPRO medium (JRH Biosciences, Cat. No. 14560). Cells were transferred directly from ExCell™ 525 to ExCell™ VPRO for the batch productions.

Fig. 1 shows that the maximum final viable cell concentration of cultures started at 1 x 10^6 cells ml^{-1} reached almost 14 x 10^6 cells ml^{-1} after 6 days (approximately 3-fold higher than batch cultures of CHO and Sp2/0), compared to cultures started at 0.3 x 10^6 ml^{-1}, which reached 10 x 10^6 cells ml^{-1} after 9 days. There is very little difference in the final antibody titres of both cultures. However, in the culture started at 1 x 10^6 ml^{-1}, approximately 600 mg L^{-1} was reached after 6 days, compared to 9 days for the cultures started at 0.3 x 10^6 ml^{-1}.

The higher cell concentrations observed in cultures started at 1 x 10^6 cells ml^{-1} compared to 0.3 x 10^5 ml^{-1} is due to the lower cell specific rate of nutrient utilisation at the higher cell concentration. The respiration rate of hybridoma cells has been shown to decrease with increasing cell density (Wohlpart et al 1990). Similarly, the cell specific rate of utilisation of a nutrient has also been shown to decrease with increasing cell concentration (Portner et al 1994, Yallop and Svendsen 2001). We have now used this information in a novel

and inventive way to form a concept for increasing attainable cell densities in a culture.

By calculating the cell specific rate of utilisation of a key nutrient each day in a batch culture and plotting these values against cell concentration, a graph can be obtained as shown in Fig. 2 for glutamine. Fig. 2 shows the relationship between the cell specific rate of glutamine utilisation (q_{Gln}) and cell concentration. From this graph, an optimum starting cell concentration can be selected based on optimal use of the available nutrients. For example, a culture starting at 0.3 x 10^6 cells ml^{-1} will reach approximately 0.5 x 10^6 ml^{-1} in 24h (average population doubling time (pdt) of this clone is 32h). The q_{Gln} value at 0.5 x 10^6 cells ml^{-1} is approximately 2.5 µmoles 10^6 $cells^{-1}$ $24h^{-1}$. The total glutamine consumed in this 24h will therefore be approximately 1.25 µmoles ml^{-1} (0.5 x 2.5). However, a culture starting at 1 x 10^6 cells ml^{-1} will reach approximately 1.5 x 10^6 ml^{-1} in 24h. The q_{Gln} value at this cell concentration is approximately 0.75 µmoles 10^6 $cells^{-1}$ $24h^{-1}$. The total glutamine consumed will therefore be approximately 1.125 µmoles ml^{-1}. The two cultures will therefore use approximately the same amount of glutamine in the first 24h.

It is therefore another object of the invention to provide a method of culturing cells, comprising starting a culture at a cell concentration where the specific nutrient utilization level is close to a minimum plateau level. This equates with around 0.8 to 2.0 x 10^6 cells/ml, preferably 0.9 - 1.5 x 10^6 cells/ml, for E1-immortalized retina cells, particularly PER.C6-derived cells. It is therefore an embodiment of the invention to subculture the cells at a seeding concentration of 0.8-2.0 x 10^6 cells/ml, preferably

$0.9\text{-}1.5 \times 10^6$ cells/ml, more preferably $0.95\text{-}1.25 \times 10^6$ cells/ml.

The advantage of this aspect of the invention is that the number of viable cells that can be obtained is higher at this higher seeding density, and higher numbers of cells are reached faster during the process. This aspect of the invention therefore is very useful for batch cultures, but can also be beneficially used in fed-batch cultures or (fed-) perfusion cultures, such as those of the present invention.

Example 2: Feed Strategies for improving antibody yields in PER.C6 derived sub-clones.

Fed-batch processes aim at increasing product yields by increasing the viable cell concentration or prolonging the production period by feeding nutrient concentrates to replenish those that are consumed. We present here a feed strategy for improving the antibody yields of PER.C6 derived sub-clones. The feed strategy can be combined with a higher starting cell density to obtain a higher final cell density at the onset of the nutrient feed and a shorter overall production process.

A basic nutrient feed concentrate consisting of glucose, phosphate, glutamine and the 15 other amino acids was prepared based on the nutrient utilisation profile of six duplicate batch cultures of clone 1 in shake-flask (see e.g. Fig. 3). Similar utilization profiles were observed for clone 2, and hence it is expected that the feed strategy described below for clone 1 will also improve yields from other clones, thereby providing a more generic strategy for fed-batch or fed-perfusion cultures of E1-immortalized cells, preferably retina cells, preferably cells derived from PER.C6 cells. The concentrate is listed in Table 1. Optionally, calcium and

three recombinant growth factors, LongR3 IGF-1, Long EGF and insulin were also added to the feed. At this point, the addition of calcium and the growth factors did not significantly influence the results that were obtained. Glycine appeared not essential for the feed, and was no longer added in later experiments. Insulin was purchased from Sigma, LongR3 IGF-1 and Long EGF were purchased from GroPep. All amino acids were purchased from Sigma. The timing and frequency of addition of the feed concentrates was varied. The time of the first addition was tested at 0, 1 and 2 days prior to nutrient exhaustion. Glucose and phosphate were used as indicators for the start of the feed. A series of bolus additions were made every two days, based on the predicted viable cell concentration. Usually, 6 feeds were provided. The concentrations of the added components as presented in Table 1 do not take into account the remaining component in the spent medium before the addition (i.e. the concentration of a component after addition into the culture medium will be higher than that provided in the Table, because before the addition the culture medium will still contain some of this component, as additions according to the invention are done before the component is completely used up by the cells).

Fig. 4 shows the effect of feeding the concentrate mix to a sub-clone of PER.C6 expressing a recombinant antibody (clone 1). Starting the feed at day 3 (two days prior to nutrient exhaustion and continuing every two days after this) resulted in a final antibody yield of approximately 800 mg L^{-1}, an increase of approximately 1.6-fold over the batch process, which gave 500 mg L^{-1}. Starting the feed at day 5 and continuing every two days after this) resulted in a similar increase in final antibody concentration.

Osmolality in the batch cultures (example 1) decreased from 280 to 240 mOsm Kg^{-1}, while in the feed cultures, it increased, eventually rising to 300-310 mOsm Kg^{-1}.

Example 3: Achieving viable cell numbers above 30 x 10^6 cells per ml and antibody yields above 500mg L^{-1} day^{-1}

Fed-batch processes may result in a build-up of toxic metabolites such as lactate and ammonia and an increase in medium osmolarity, which eventually limit the viable cell concentration and the length of the process, thus impacting on product yields. A possible alternative to a fed-batch process is a perfusion process, where high cell concentrations can be maintained by a continual medium exchange and a cell bleed (removing a certain percentage of the cells population). A possible drawback with such a process is a relatively low product concentration due to the large volumes of medium that are required, the relatively low cell viability often encountered and the relatively high level of complexity to operate such a system. It is therefore only advantageous to operate perfusion processes if very high viable cell concentrations and/or specific productivities can be maintained.

We present here the attainment of a viable cell concentration above 30 x 10^6 ml^{-1} and antibody yields of above 600 mg L^{-1} $24h^{-1}$ in shake flask cultures with one medium volume exchange per day.

Logarithmic cultures of antibody producing PER.C6 cells, cultured in shake flask with ExCell™ 525 were transferred into shake flasks containing ExCell™ VPRO at a starting cell number 1 x 10^6 cells/ml (other starting cell concentrations gave similar results).. Medium replacement by centrifugation (one volume per day) was started at day 3-5. No cell bleed was

operated. Samples for metabolite analysis, antibody quantification and cell counts were taken every day and stored at −20°C.

Fig. 5 shows that a viable cell number of up to 50 x 10^6 ml^{-1} and an antibody yield of 500-750 mg L^{-1} $24h^{-1}$ was maintained for at least 5 days without a cell bleed, for two independent antibody producing cell clones. Viability of the cells was around 80-90%. These high cell densities are approximately 3-fold higher than is generally achievable with other cell lines like CHO and Sp2/0, and hence retina cells that are immortalized with adenovirus E1 sequences, such as PER.C6 cells, are very suitable for perfusion processes. A cell bleed will improve the length of the process, and therefore an optimized system may include one or more cell bleed steps.

Up to 50 x 10^6 cells per ml, with a viability of around 80-90%, could be maintained for at least 5 days with one complete medium change every two days. With this strategy, many of the nutrients became depleted on the second day. The medium is therefore preferably changed daily. In a perfusion process, this could translate into a change of about 1-3 volumes/day. This is near the typical range in a standard perfusion system, where the medium is changed at about 0.5 to 2 volumes/day. The somewhat higher values for the cells according to the invention are due to the very high cell concentrations with the cells of the invention in a perfusion system. When cell concentrations of more than 30 x 10^6 cells/ml according to the invention are preferred, the medium exchange should at least be 0.5 culture volumes/day, preferably at least 1 culture volume/day. Failure to supply the nutrients (here via the culture medium) in sufficient concentration leads to cell death. The daily medium change results in higher

viable cell densities (up to 50 x 10^6 cells/ml with daily medium change vs. 10 x 10^6 cells/ml without daily medium change, see Figs 1 and 4). Furthermore, with a daily medium exchange, the cells give similar product yields in one day as achieved in a batch process of 8-13 days.

Example 4: Feed Strategies for further improving antibody yields in PER.C6 derived sub-clones.

The provision of a balanced nutrient feed extends to components such as vitamins, trace elements and lipids. Concentrates (10x or 50x, both worked) of ExCell VPRO vitamins, inorganic salts, trace elements, growth factors, lipids and plant hydrolysates were obtained from JRH Biosciences and added together with the basic feed concentrate (minus calcium and growth factors) described in example 2. The ExCell VPRO concentrates were added to give a final concentration of 0.25X.

Fig. 6 shows the results of this modified feed on the growth (Fig. 6A) and antibody yields (Fig. 6B) of clone 1 in shake-flask versus a batch control. The results were obtained by starting the feed at day 3 (48h prior to nutrient depletion). Starting the feed at day 5 (day of nutrient depletion) gave similar results. The viable cell number was maintained for significantly longer than the batch control and antibody yields increased 2.0-fold from 0.5 g L^{-1} in the batch to 1.0 g L^{-1} in the fed-batch process.

Spent medium analysis of these feed experiments identified a change in the cell specific rates of utilization of some of the amino acids, which appeared to be de to the addition of the VPRO concentrates. The amino acid concentrate listed in Example 2 was therefore modified as shown in Table 2. The feed

was started 48h prior to nutrient depletion and additions were made every two days. Usually, 6 feeds were provided. Again, the concentrations of the added components as presented in the Table do not take into account the remaining component in the spent medium before the addition.
For the first feed addition, increased concentrations of Glutamine and Serine were used as compared to the subsequent feeds (see Table 2). Phosphate and glucose were used as markers to determine the start of the feed. Clones 1 and 2 were used in this experiment.
Experiments were carried out in shake-flask and bioreactor. Shake flask experiments were carried out as described. Bioreactor experiments were initiated by inoculating a 3L bioreactor (Applikon, 2L working volume) with cells from a logarithmic pre-culture grown in shake flask. The pre-culture and bioreactor experiments were performed in ExCell VPRO (JRH Biosciences). The split ratio for inoculation into the bioreactor was at least 1:6, and the seeding cell concentration was about 0.3 x 10^6 cells/ml.

Results

Fig. 7 shows the results of the modified feed on clone 1 in bioreactor versus a batch control. The maximum viable cell number reached 10-12x10^6 ml^{-1} and viable cell numbers were maintained between 8 and 10x10^6 cells ml^{-1} until the end of the culture at day 19 (Fig. 7A). Antibody yields increased 3-fold from 0.4 g L^{-1} in the batch to 1.3 g L^{-1} in the fed-batch process (Fig. 7B).
Osmolality and ammonia reached 430 mOsm Kg^{-1} and 16 mmoles L^{-1} respectively in these feed cultures, levels that have been reported as having negative effects on culture performance and product quality. It may therefore be that the decrease in

viable cell numbers observed towards the end of the process was due at least in part to these factors.
Fig. 8 shows the results of the feed strategy on clone 2 in 2L bioreactors. Maximum viable cell numbers reached 10-11 x 10^6 ml^{-1} and 7-9 x 10^6 ml^{-1} were maintained until the end of the culture at 19 days. Antibody yields were increased 3-fold from 0.5 g L^{-1} to 1.5 g L^{-1}.
A third clone expressing another, again unrelated, antibody was subjected to the same batch process and the fed-batch process with the same feed strategy. Fig. 10 shows the results of the feed strategy on this clone 3 in shake flask. Maximum viable cell numbers reached 14 x 10^6 ml^{-1} and 10-12 x 10^6 ml^{-1} were maintained until the end of the culture on day 17. Antibody yields were increased 3-fold from 0.7 g L^{-1} to 2.1 g L^{-1}.

The feed strategy therefore improves the yield for different clones that each express a different antibody, indicating that the process according to the invention is generically applicable.
It is therefore an aspect of the invention to provide a process comprising the feed strategy according to the invention, wherein the yield of a produced protein is increased at least 1.5 x, preferably at least 2 x, more preferably at least 2.5 x, still more preferably at least 3x over the yield in the batch process.

The specific productivity (q_{Ab}) of the cells used in the present invention was approximately between 12-18 pg antibody/cell/day. In some instances the q_{Ab} was around 10 pg antibody/cell/day, and in other instances values up to about 25 pg antibody/cell/day were observed with the cells and

methods of the present invention. In the batch cultures this decreased significantly before maximum cell numbers were reached, coinciding with depletion of nutrients, which was approximately after 7 days, whereas in fed-batch cultures this specific productivity was kept at this level until 2-3 days after the last feed addition, which amounts to around 16-18 days, according to a process of the invention.

Product Quality

In the experiments described above, product quality was checked by various methods, including iso-electric focusing, SDS-PAGE, MALDI-TOF mass spectrometry and HPAEC-PAD. In all cases the produced antibody basically showed a human-type glycosylation and the structural integrity of the produced antibodies was very good, irrespective of the process used, and very similar to that reported in (Jones et al, 2003), where both cell numbers and product yields were lower. Therefore, the increased yields obtainable by processes of the invention were not obtained at the cost of a significant decrease in product quality.
Protein A purified IgG produced from batch and fed-batch cultures was analysed by MALDI-MS. Material produced by PER.C6 cells from batch cultures showed a galactosylation profile similar to that shown by IgG purified from human serum and no hybrid or high mannose structures were identified in either batch or fed-batch produced material. The average percentage of glycans terminating in 0, 1 and 2 galactose residues (G0:G1:G2) from all the batch cultures tested was 29, 54 and 17 % respectively. This can be compared to CHO and hybridoma produced antibody, which is often predominantly in the G0 form. For example, Hills et al (1999) reported a

galactosylation profile (G0:G1:G2) for an antibody produced in NS0 and CHO cells.
Antibody produced in the fed-batch process showed a reduced level of galactosylation compared to the batch (Fig. 9). The percentage of G0 glycoforms increased from 29 to 49%, while the G1 and G2 glycoforms decreased from 54% and 17% to 42% and 9% respectively. This decrease in galactosylation was probably due to the high (up to 16 mM) ammonia concentrations at the end of the fed-batch cultures. However, the level of galactosylation in the antibody produced by the fed-batch process in PER.C6 cells was still higher than typically seen in batch-produced antibodies from CHO for example (Hills et al 1999). Isoelectric focusing (IEF) and SDS-PAGE revealed no significant differences between the material produced by batch or fed-batch cultures (data not shown) and in all cases, aggregation was below 3%.
Despite relatively low $Y_{amm/gln}$ values, the high viable cell concentrations resulted in a supply of glutamine in the feed such that the ammonia accumulated up to 16 mmoles L^{-1}. Whilst this did not result in a drop in the viable cell concentration, batch cultures initiated in the presence of NH_4Cl showed that concentrations above 9 mmole L^{-1} negatively affected growth rates and maximum cell concentrations. Furthermore, glycosylation was also somewhat affected (see Fig. 9). It may therefore be beneficial to reduce ammonia accumulation, e.g. according to a method described below.

Two areas for attention in the process described so far are the high levels of ammonia and osmolality. A large contributor to the increase in osmolality came from the VPRO (medium) concentrates. An approach to reduce this osmolality is therefore to identify which of the medium component groups

(vitamins, trace elements, inorganic salts, growth factors etc) are important to culture performance and remove those that are not important. This should benefit the process not only by reducing the osmolality of the feed but also by removing any potentially deleterious components and by allowing the optimization of addition of the most important components. It would also reduce the cost of the feed. Reduction in ammonia accumulation may be achieved by more strictly controlling glutamine addition. This can be done based on the calculations of the specific consumption and cell numbers as described supra. This can be achieved by continuously pumping in glutamine at an appropriate rate, matched to the viable cell concentration and the cell specific rate of utilization, so that residual glutamine concentrations in the medium are maintained at a constant low level, such as between 0.2 and 1.5 mM, preferably between 0.5 and 1.0 mM. Another approach that may be possible for the cells according to the invention is the removal of glutamine from the feed when the ammonia concentration reaches a certain point -e.g. in one or more of the feeds subsequent to the first feed- so that the cells are forced to switch to glutamine synthesis using ammonia and glutamate and the glutamine synthetase pathway. This approach is not generally possible for cell types such as BHK and CHO as glutamine depletion often results in rapid and widespread cell death and transfer to glutamine-free conditions often requires a period of adaptation. However, in batch cultures of the cells according to the present invention, the viable cell concentration continued to increase for two days after the depletion of glutamine and culture viability was not significantly affected, suggesting that there may be sufficient flux through the glutamine synthetase pathway at least to maintain the culture.

Spent medium analysis of the most optimized fed-batch culture (examples in Fig. 7, 8) showed that only cystine was depleted during the process. A further modification of the amino acid feed according to the invention is therefore an increase in the cystine concentration, e.g. to 0.3-0.35 mmoles/l or even up to 0.6 mmoles/l for every 10 x 10^6 cells/ml.

Example 5: improved (fed-)batch process.

Feed concentrates developed for fed-batch processes may also be used to supplement culture media for use in an improved batch process. Supplementing a culture medium with at least one of the feed additions from a fed-batch process has been shown by others to improve batch yields. A similar approach of supplementing culture media with feed concentrates may also be used to reduce the number of feed additions during a fed-batch process, thereby simplifying the process, as also shown by others.

The present invention discloses feed strategies for cells that have been immortalized by adenovirus E1 sequences, such as PER.C6 cells. It is shown herein which components become limiting in a fed-batch process, and the amounts of as well as the ratio between the components that can be added to improve yields in a fed-batch process are disclosed herein. This information is used in this example to provide an improved batch process. It is assumed that such a culture will contain about 10 x 10^6 cells/ml, as this is around the number of cells that has been observed in the batch and fed-batch cultures of the invention. In the fed-batch experiments, 6 feeds were added, with concentrations of the components as in Tables 1 or 2. The addition of 10%-60% of the total (i.e. the total of all 6 feeds together) feed, preferably 20%-40% of the total feed, results in an improved batch process, because the nutrients

will become depleted later during the culture, and hence the yields will go up because of prolonged productivity compared to the straight batch process disclosed above, where no additions are made to the culture medium. The components can be added directly to the culture medium at any stage prior to depletion of nutrients from the medium, but are preferably added prior to start of the culture so that no other additions have to be made during the process (improved batch process), which makes the process very simple. Of course, this may be combined with extra additions of certain components later during the process (fed-batch process), in which case less additions have to be made to make the process than in the fed-batch process disclosed above, thereby providing a simpler fed-batch process. It is therefore another embodiment of the invention to provide a method for producing a product in cells immortalized by adenovirus E1 sequences, wherein said cells are cultured in a culture medium, characterized in that the following components are added to the culture medium in the following amounts: glucose (3.6 - 21.6 mmoles/l, preferably 7.2 - 14.4 mmoles/l), glutamine (6.8 - 40.9 mmoles/l, preferably 13.6 - 27.2 mmoles/l), leucine (0.40 - 2.4 mmoles/l, preferably 0.79 - 1.6 mmoles/l), serine (2.31 - 13.9 mmoles/l, preferably 4.62 - 9.24 mmoles/l), isoleucine (0.3 - 1.8 mmoles/l, preferably 0.6 - 1.2 mmoles/l), arginine (0.28 - 1.66 mmoles/l, preferably 0.55 - 1.10 mmoles/l), methionine (0.14 - 0.83 mmoles/l, preferably 0.28 - 0.55 mmoles/l), cystine (0.15 - 0.9 mmoles/l, preferably 0.3 - 0.6 mmoles/l), valine (0.27 - 1.62 mmoles/l, preferably 0.54 - 1.08 mmoles/l), lysine (0.26 - 1.58 mmoles/l, preferably 0.53- 1.06 mmoles/l), threonine (0.18 - 1.08 mmoles/l, preferably 0.36 - 0.72 mmoles/l), asparagine (0.06 - 0.36 mmoles/l, preferably 0.12 - 0.24 mmoles/l), tyrosine (0.078 - 0.47 mmoles/l,

preferably 0.16 - 0.31 mmoles/l), histidine (0.06 - 0.36 mmoles/l, preferably 0.12 - 0.24 mmoles/l), phenylalanine (0.012 - 0.072 mmoles/l, preferably 0.024 - 0.048 mmoles/l), tryptophan (0.036 - 0.22 mmoles/l, preferably 0.072 - 0.14 mmoles/l) and phosphate (0.45 - 2.7 mmoles/l, preferably 0.9 - 1.8 mmoles/l). The amounts between brackets are 10%-60%, preferably 20%-40%, of the amounts of 6x the feeds of Table 2. Preferably, also culture medium concentrate (10x, 50x, or other suitable concentrates can be used) is added to an end concentration of between 0.15x - 0.9x, preferably between 0.3x - 0.6x. Preferably the culture medium in these embodiments is ExCell VPRO medium. An amount of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 or 4 single feeds (a single feed being an amount as disclosed in Table 1 or 2) is added to culture medium, and simple batch processes for culturing the cells at around 10 x 10^6 cells/ml and producing product (e.g. antibody) according to the invention are performed with the thus fortified media, to determine the optimum amount of component additions. Improved batch processes giving the highest product yields are expected when about 20%-40% of the total feed of a fed-batch process according to the invention are provided to the culture medium prior to culturing, i.e. somewhere between 1 to 2.5 single feeds. Of course, more fine-tuning of the amount is possible once a beneficial range of added components is established by these experiments. Of course, when the cell numbers are different, the component addition can again be adapted. For instance, if the cells are cultured at a density of only 5 x 10^6 cells/ml, addition of an amount of only half the amount above would be required, as is clear to the person skilled in the art.

Table 1

Components	**Final Concentration (after addition) (per 10 x 10^6 cells/ml) (mmoles L^{-1})**
Glucose	6.00
Glutamine	1.75
Leucine	0.60
Serine	0.55
Isoleucine	0.45
Arginine	0.42
Methionine	0.23
Cystine	0.14
Valine	0.45
Lysine	0.40
Threonine	0.33
Glycine	0.33
Asparagine	0.15
Tyrosine	0.14
Histidine	0.11
Penylalanine	0.10
Tryptophan	0.02
Phosphate	0.70
Calcium	0.02*
LongR3 IGF-1	50 ug/L*
Long EGF	50 ug/L*
Insulin	20 ug/L*

*optionally present

Table 2

Components	**Final Concentration (after addition) (per 10 x 10^6 cells/ml) (mmoles L^{-1})**	
	First Feed	**Subsequent Feeds**
Glucose	6.00	6.00
Glutamine	2.60	1.75
Leucine	0.66	0.66
Serine	1.10	0.55
Isoleucine	0.50	0.50
Arginine	0.46	0.46
Methionine	0.23	0.23
Cystine	0.25	0.23
Valine	0.45	0.45
Lysine	0.44	0.44
Threonine	0.30	0.30
Asparagine	0.10	0.10
Tyrosine	0.13	0.13
Histidine	0.10	0.10
Penylalanine	0.02	0.02
Tryptophan	0.06	0.06
Phosphate	0.75	0.75
10X VPRO Concentrate	0.25X	0.25X

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CLAIMS

1. A method for the culturing of cells immortalized by adenovirus E1 sequences, said cells capable of growing in suspension, comprising the steps of:
a) determining at least once during the culturing of the cells the concentration of at least one medium component selected from the group consisting of glucose, glutamine phosphate, leucine, serine, isoleucine, arginine, methionine, cystine, valine, lysine, threonine and glycine,
b) adding components to the medium during the culturing of the cells at or prior to the depletion of at least one of the components of which the concentration was determined in step a), wherein
the components added at least comprise glucose, glutamine, phosphate, leucine, serine, isoleucine, arginine, methionine and cystine.

2. A method according to claim 1, wherein the at least one medium component of which the concentration is determined in step a) are chosen from the group consisting of glucose, glutamine, phosphate, leucine, serine, isoleucine, arginine, methionine and cystine.

3. A method according to claim 1 or 2, wherein in step a) the concentration is determined of at least two of said medium components.

4. A method according to any one of claims 1-3, wherein the components added in step b) further comprise one or more of valine, lysine, threonine, glycine, asparagine,

tyrosine, histidine, phenylalanine, tryptophan, phosphate, calcium, LongR3 IGF-1, Long EGF and insulin.

5. A method according to any one of claims 1-4, wherein the components in step b) are added in an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of between 4.0 and 8.0 for glucose, 0.44 and 0.88 for leucine, 0.37 and 1.47 for serine, 0.33 and 0.67 for isoleucine, 0.31 and 0.61 for arginine, 0.15 and 0.31 for methionine, and 0.1 and 0.6 for cystine.

6. A method according to claim 5, wherein in step b) glutamine is further added to an end concentration of 1.17 and 3.47 mmoles/l of freshly added glutamine per 10 x 10^6 cells/ml.

7. A method according to claim 6, wherein the components in step b) are added more than one time, further characterized in that as a result of the first addition the end concentration of freshly added glutamine is higher than as a result of a subsequent addition.

8. A method according to claim 5, wherein glutamine is further added essentially continuously such that the residual concentration of glutamine in the medium is maintained between 0.2 and 1.5 mM, preferably between 0.5 and 1.0 mM.

9. A method according to any one of claims 5-8, wherein in step b) the following components are further added to an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of between 0.3 and 0.6 for valine,

0.29 and 0.59 for lysine, 0.2 and 0.4 for threonine.

10. A method according to claim 9, wherein in step b) the following components are further added to an end concentration in mmoles/l of freshly added component per 10 x 10^6 cells/ml of between 0.067 and 0.13 for asparagine, 0.087 and 0.17 for tyrosine, 0.067 and 0.13 for histidine, 0.013 and 0.027 for phenylalanine, and 0.04 and 0.08 for tryptophan.

11. A method according to any one of claims 1-10, wherein the addition of the components in step b) is performed at between 48 hours and just prior to depletion of at least one of the medium components the concentration of which was determined in the previous step.

12. A method according to any one of claims 1-11, wherein said steps are repeated at least once.

13. A method according to any one of claims 1-12, wherein said cells are derived from retina cells.

14. A method according to claim 13, wherein said cells are derived from human embryonic retina cells as represented by the cells deposited under ECACC no. 96022940.

15. A method according to any one of claims 1-14, wherein the cells are grown to a cell density of at least 9 x 10^6 per ml.

16. A method according to any one of claims 1-15, wherein the cells produce a product that is harvested.

17. A method according to claim 16, wherein said product is a recombinant protein.

18. A method according to claim 17, wherein said recombinant protein is an immunoglobulin that is secreted into the culture medium to a level of at least 500 mg per liter.

19. A method according to claim 18, wherein said recombinant protein is an immunoglobulin that is secreted into the culture medium to a level of at least 1000 mg per liter.

20. A method according to anyone of the preceding claims, wherein the cells are in suspension during culturing.

21. A culture of cells immortalized by adenovirus E1 sequences, characterized in that said culture comprises at least 10 x 10^6 cells/ml.

22. A culture of cells according to claim 21, characterized in that said culture comprises at least 12 x 10^6 cells/ml.

23. A culture of cells according to claim 22, characterized in that said culture comprises at least 15 x 10^6 cells/ml.

24. A culture of cells according to claim 23, characterized in that said culture comprises at least 20 x 10^6 cells/ml.

25. A culture of cells according to claim 24, characterized in that said culture comprises at least 30 x 10^6 cells/ml.

26. A culture of cells according to claim 25, characterized in that said culture comprises at least 40 x 10^6 cells/ml.

27. A culture of cells according to any one of claims 21-26, wherein at least 80% of the cells are viable.

28. A culture of cells according to any one of claims 21-27, wherein culture medium is exchanged at a rate of about 0.2-3 culture volumes/day.

29. A culture of cells according to any one of claims 21-28, wherein cells in said culture express a recombinant protein.

30. A culture of cells according to claim 29, wherein said cells produce at least 10 pg protein/cell/day.

31. A culture of cells according to claim 21, wherein said culture is a batch culture and wherein cells in said culture express a recombinant immunoglobulin with a yield of at least 500 mg/l.

32. A culture of cells according to claim 30, wherein said yield is at least 700 mg/l.

33. A culture of cells according to claim 29 or claim 30, wherein said culture is a perfusion or fed-perfusion

culture and wherein said recombinant protein is an immunoglobulin that is expressed with a yield of at least 150 mg/l/day.

34. A culture of cells according to any one of claims 21-33, wherein said cells are derived from human embryonic retina cells as represented by the cells deposited under ECACC no. 96022940.

35. A culture of cells according to any one of claims 21-34, wherein said culture is a suspension culture.

36. A method for producing a product in cells immortalized by adenovirus E1 sequences, said cells being in a culture medium, wherein said product is chosen from the group consisting of a recombinant protein, a virus, and a recombinant adenovirus with a deletion in the E1 region, characterized in that
at least Glutamine, Glucose, Phosphate, Leucine, Serine, Isoleucine, Arginine, Methionine and Cystine are added to the culture medium.

37. A method according to claim 36, further characterized in that said cells reach a cell concentration of at least 20 x 10^6, preferably at least 30 x 10^6 viable cells/ml at least part of the time in said process.

38. A method for producing a product in cells immortalized by adenovirus E1 sequences, wherein said cells are cultured in a culture medium, characterized in that the following components are added to the culture medium per liter: 3,6-21.6 mmoles glucose, 6.8-40.9 mmoles

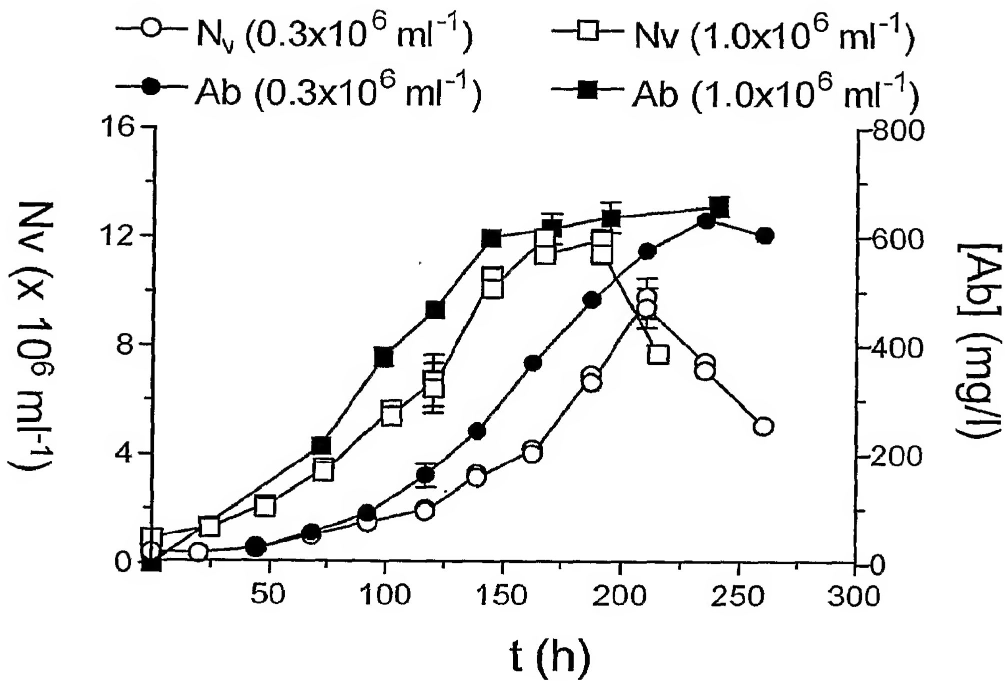
glutamine, 0.40-2.4 mmoles leucine, 2.31-13.9 mmoles serine, 0.3-1.8 mmoles isoleucine, 0.28-1.66 mmoles arginine, 0.14-0.83 mmoles methionine, 0.15-0.9 mmoles cystine, 0.27-1.62 mmoles valine, 0.26-1.58 mmoles lysine, 0.18-1.08 mmoles threonine, 0.06-0.36 mmoles asparagine, 0.078-0.47 mmoles tyrosine, 0.06-0.36 mmoles histidine, 0.012-0.072 mmoles phenylalanine, 0.036-0.22 mmoles tryptophan and 0.45-2.7 mmoles phosphate.

39. A method according to claim 38, wherein the amounts of the components added to the culture medium per liter are: 7.2-14.4 mmoles glucose, 13.6-27.2 mmoles glutamine, 0.79-1.6 mmoles leucine, 4.62-9.24 mmoles serine, 0.6-1.2 mmoles isoleucine, 0.55-1.10 mmoles arginine, 0.28-0.55 mmoles methionine, 0.3-0.6 mmoles cystine, 0.54-1.08 mmoles valine, 0.53-1.06 mmoles lysine, 0.36-0.72 mmoles threonine, 0.12-0.24 mmoles asparagine, 0.16-0.31 mmoles tyrosine, 0.12-0.24 mmoles histidine, 0.024-0.048 mmoles phenylalanine, 0.072-0.14 mmoles tryptophan and 0.9-1.8 mmoles phosphate.

40. A method according to claim 38 or claim 39, wherein further culture medium concentrate is added to an end concentration of between 0.15x - 0.9x, preferably between 0.3x - 0.6x.

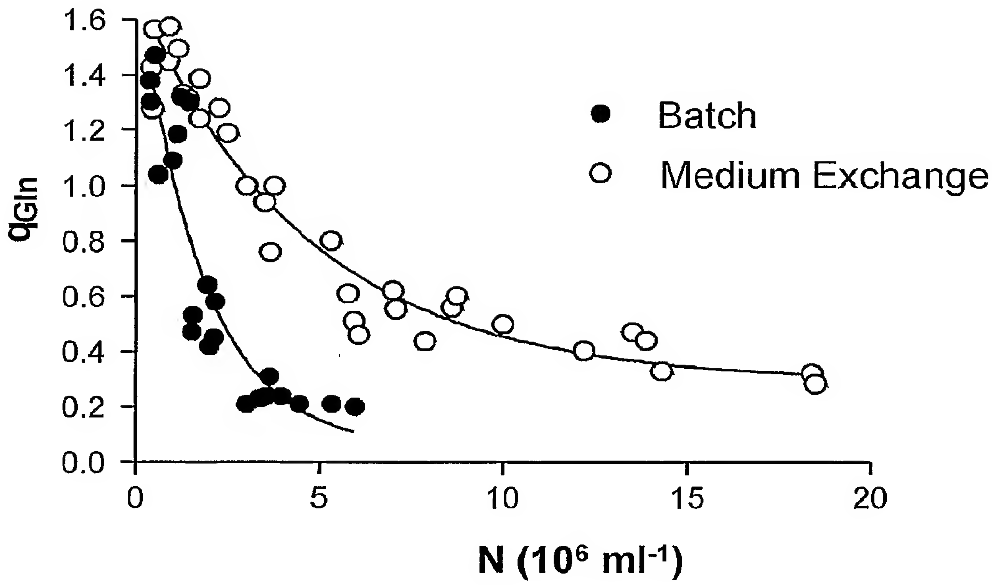
41. A method according to any one of claims 38-40, wherein said components are added to the culture medium prior to culturing the cells.

Fig 1



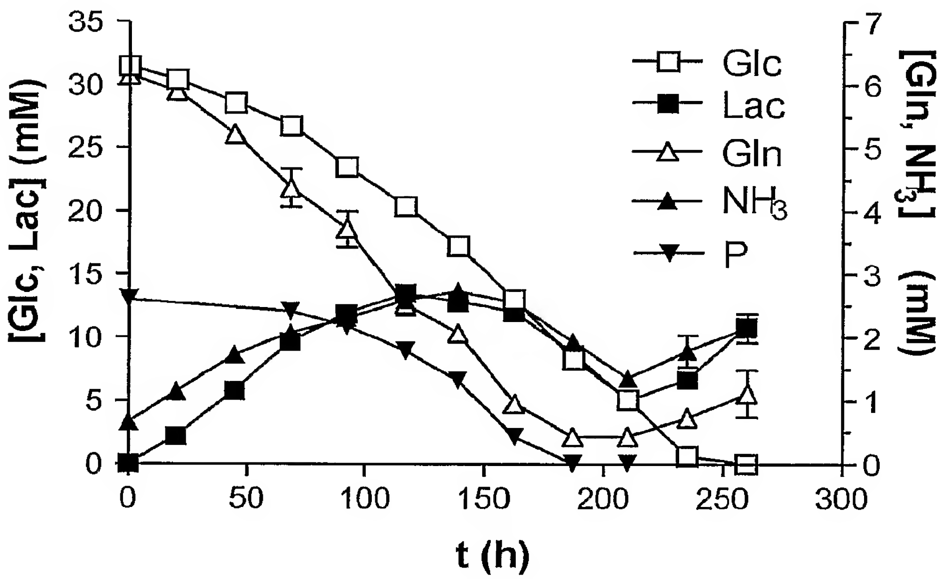
Nv (0.3x10⁶ ml⁻¹)
Nv (1.0x10⁶ ml⁻¹)
Ab (0.3x10⁶ ml⁻¹)
Ab (1.0x10⁶ ml⁻¹)
Nv (x 10⁶ ml⁻¹)
[Ab] (mg/l)
t (h)
0
4
8
12
16
0
200
400
600
800
50
100
150
200
250
300

Fig. 2



1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
q_{Gln}
0
5
10
15
20
N (10^6 ml^{-1})
Batch
Medium Exchange

Fig.3A



35
30
25
20
15
10
5
0
[Glc, Lac] (mM)
0
50
100
150
200
250
300
t (h)
7
6
5
4
3
2
1
0
[Gln, NH3] (mM)
Glc
Lac
Gln
NH3
P

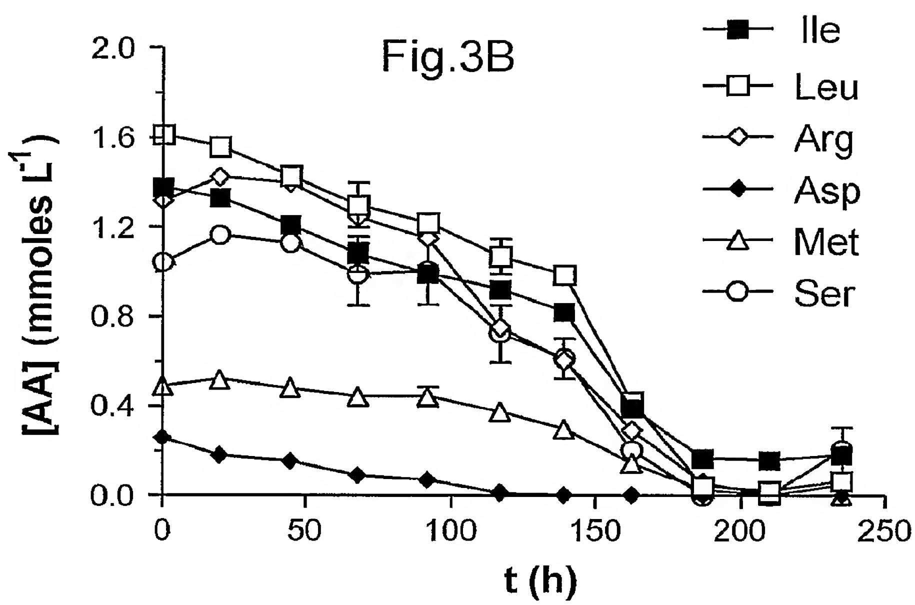


Fig.3B
Ile
Leu
Arg
Asp
Met
Ser
2.0
1.6
1.2
0.8
0.4
0.0
[AA] (mmoles L-1)
0
50
100
150
200
250
t (h)

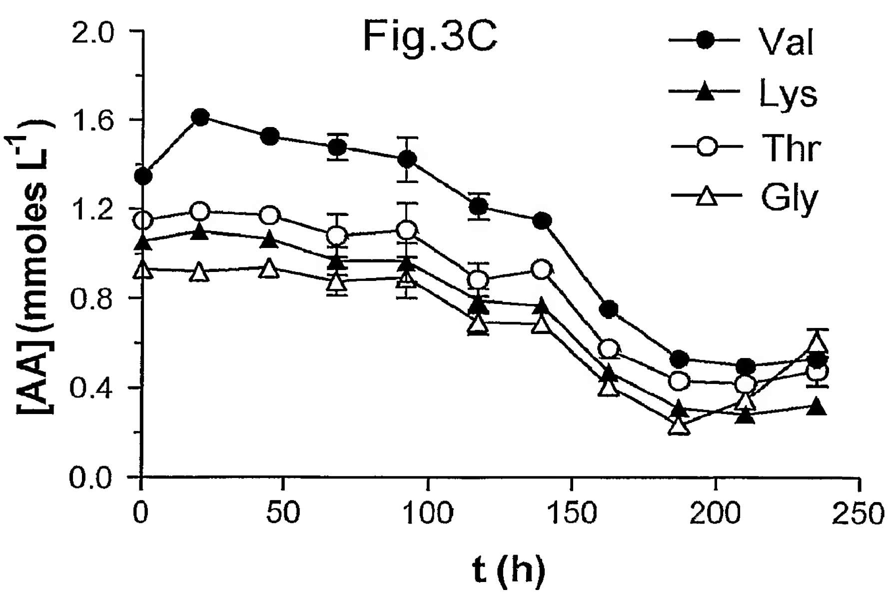


Fig.3C
Val
Lys
Thr
Gly
2.0
1.6
1.2
0.8
0.4
0.0
[AA] (mmoles L-1)
0
50
100
150
200
250
t (h)

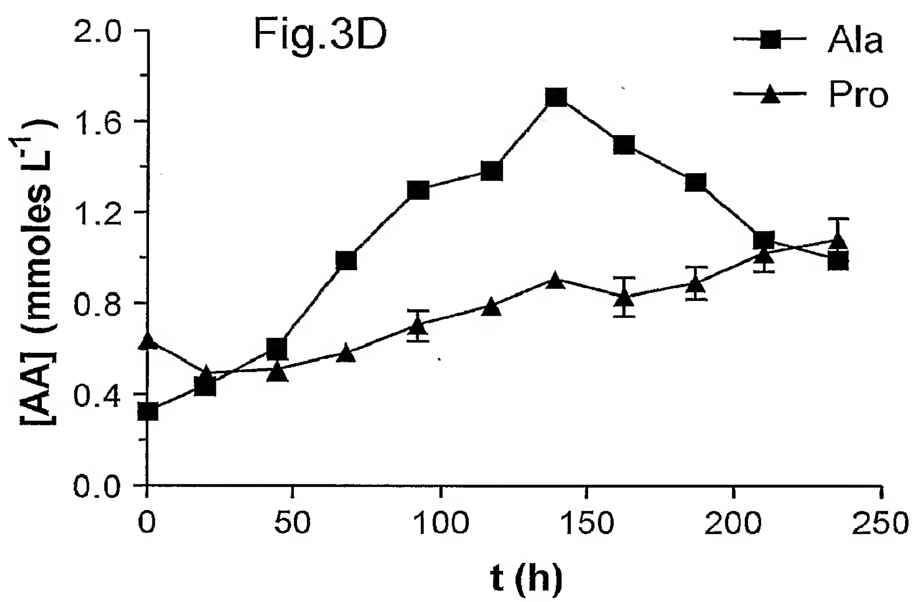


Fig.3D
Ala
Pro
[AA] (mmoles L^{-1})
2.0
1.6
1.2
0.8
0.4
0.0
0
50
100
150
200
250
t (h)

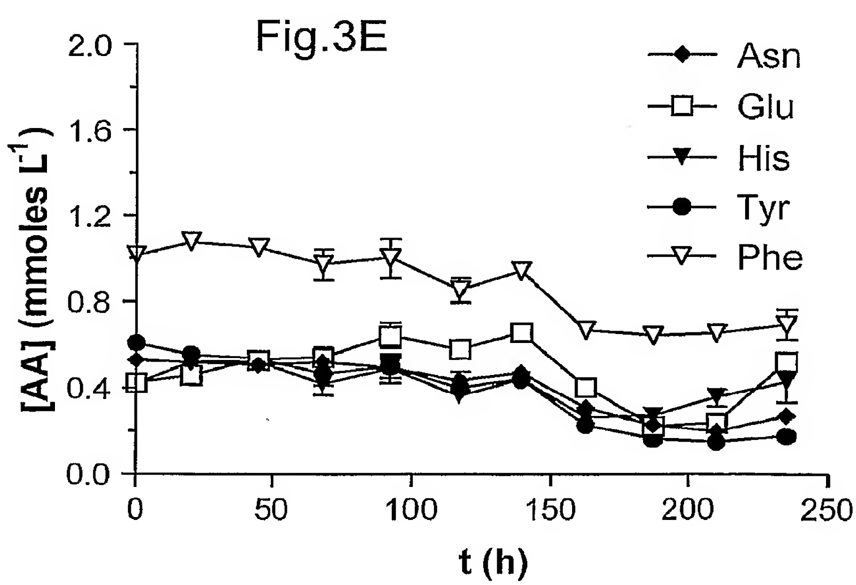


Fig.3E
Asn
Glu
His
Tyr
Phe
[AA] (mmoles L^{-1})
2.0
1.6
1.2
0.8
0.4
0.0
0
50
100
150
200
250
t (h)

Fig. 4

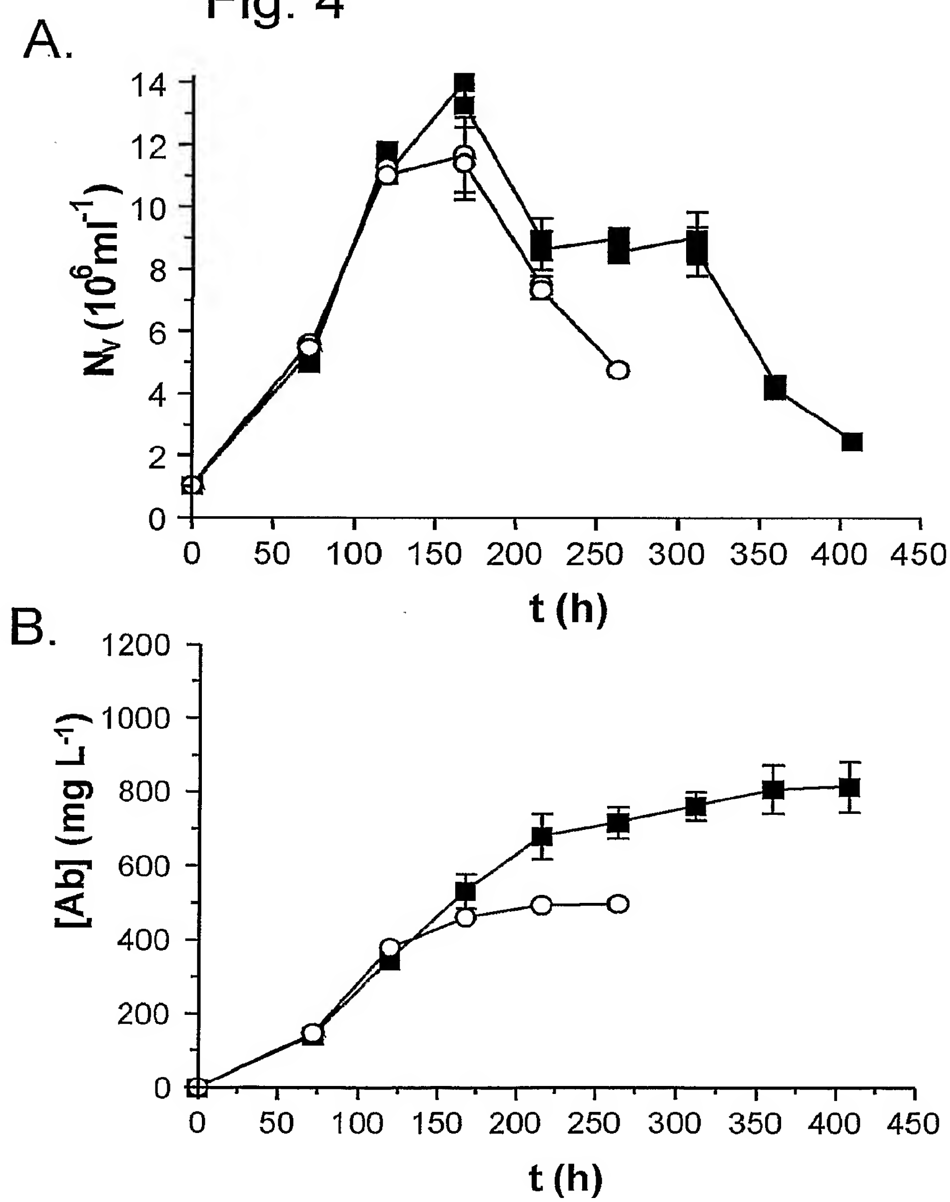

A.
14
12
10
8
6
4
2
0
N_V (10^6ml^{-1})
0 50 100 150 200 250 300 350 400 450
t (h)
B.
1200
1000
800
600
400
200
0
[Ab] (mg L^{-1})
0 50 100 150 200 250 300 350 400 450
t (h)

Fig. 5

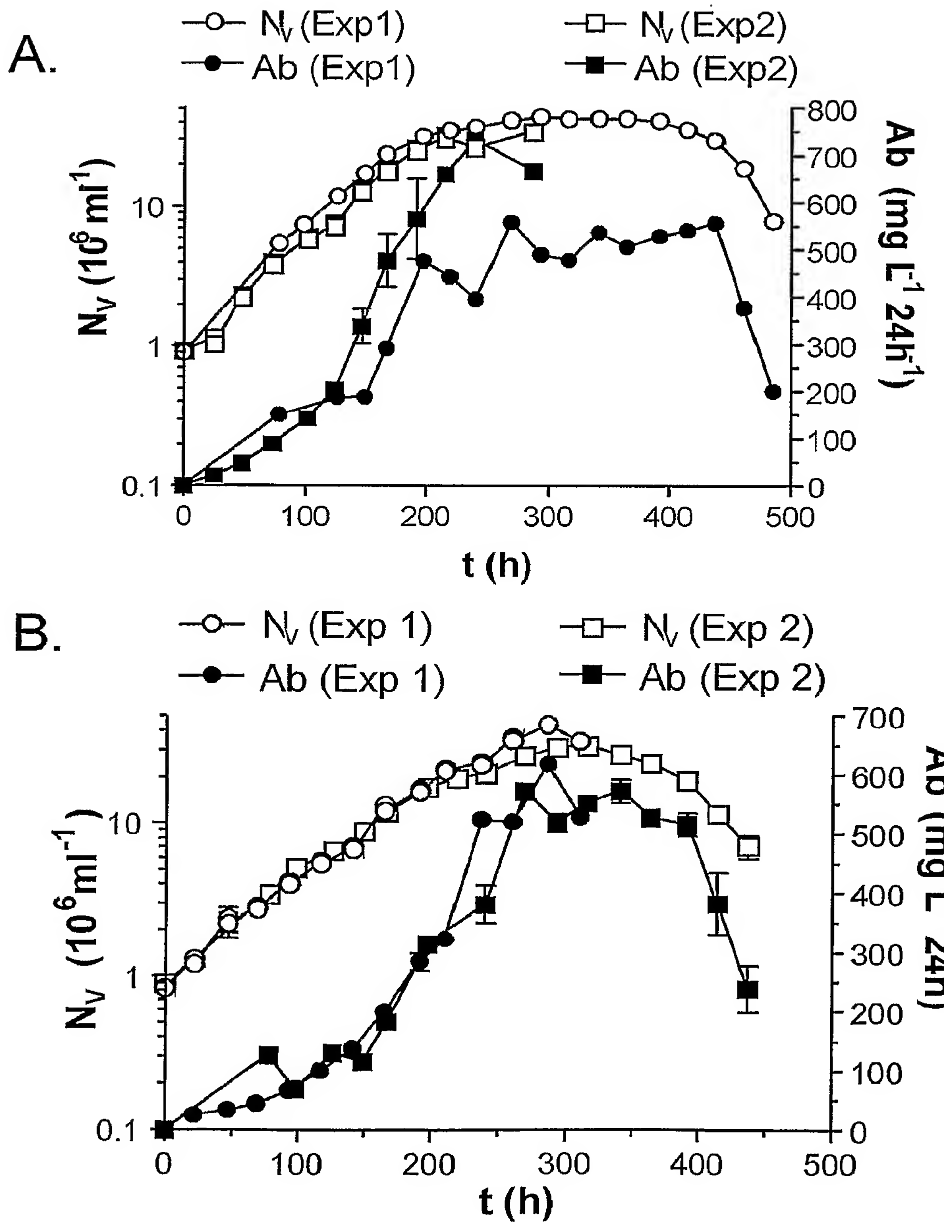

A.
Nv (Exp1)
Nv (Exp2)
Ab (Exp1)
Ab (Exp2)
Nv (10^6 ml^-1)
Ab (mg L^-1 24h^-1)
t (h)
B.
Nv (Exp 1)
Nv (Exp 2)
Ab (Exp 1)
Ab (Exp 2)
Nv (10^6 ml^-1)
Ab (mg L^-1 24h)
t (h)

Fig. 6

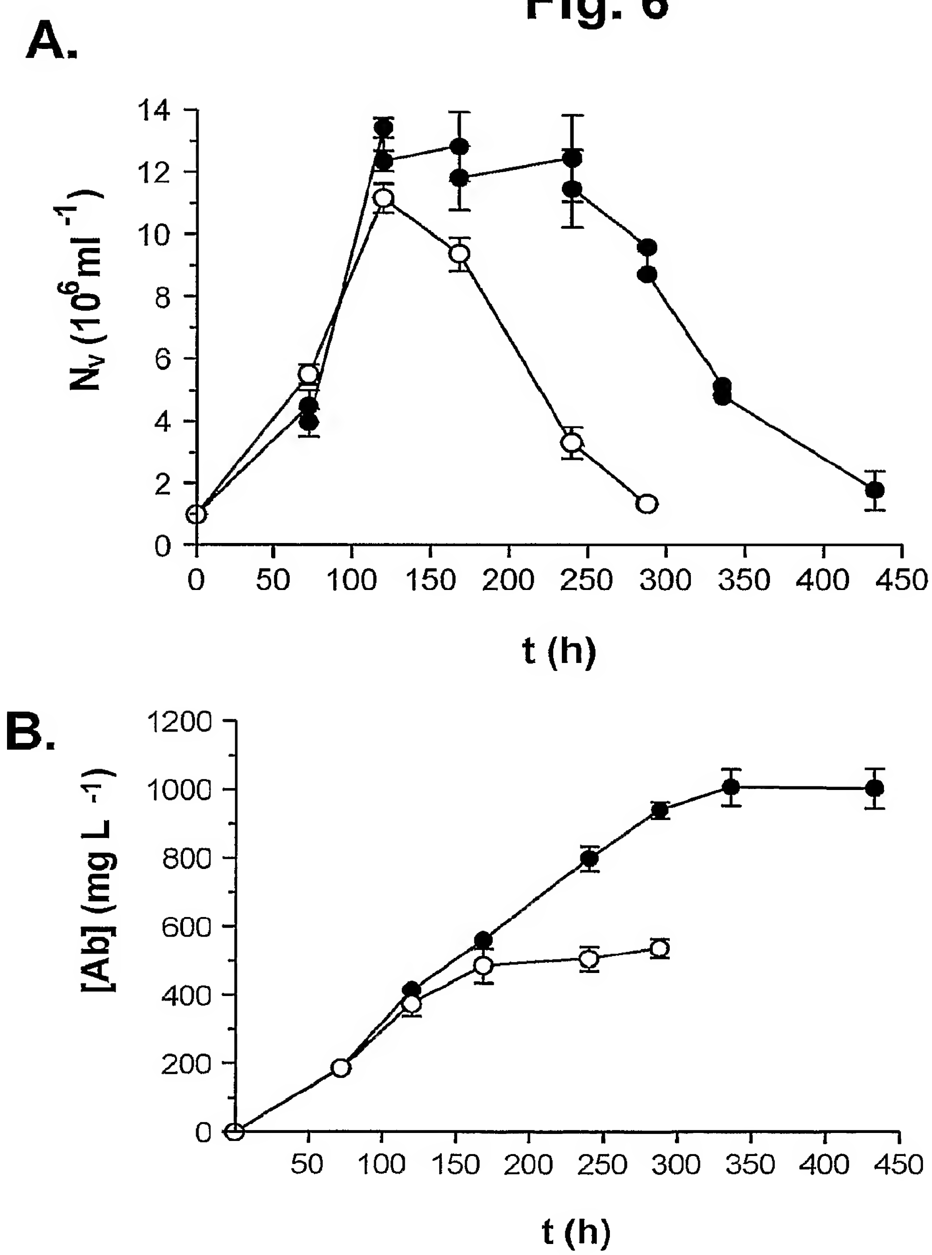
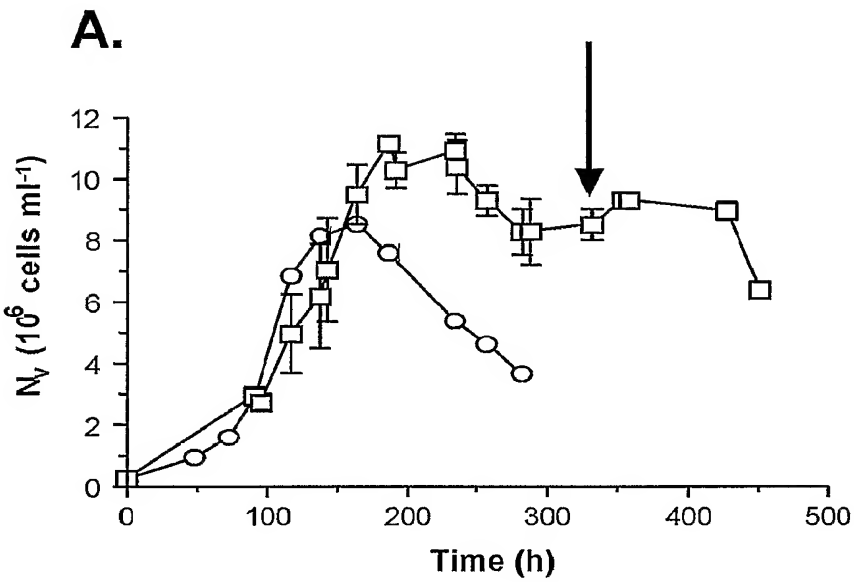

A.
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
400
450
N_V (10^6ml $^{-1}$)
t (h)
B.
1200
1000
800
600
400
200
0
50
100
150
200
250
300
350
400
450
[Ab] (mg L $^{-1}$)
t (h)

Fig. 7


A.
12
10
8
6
4
2
0
N_V (10^6 cells ml^{-1})
0
100
200
300
400
500
Time (h)

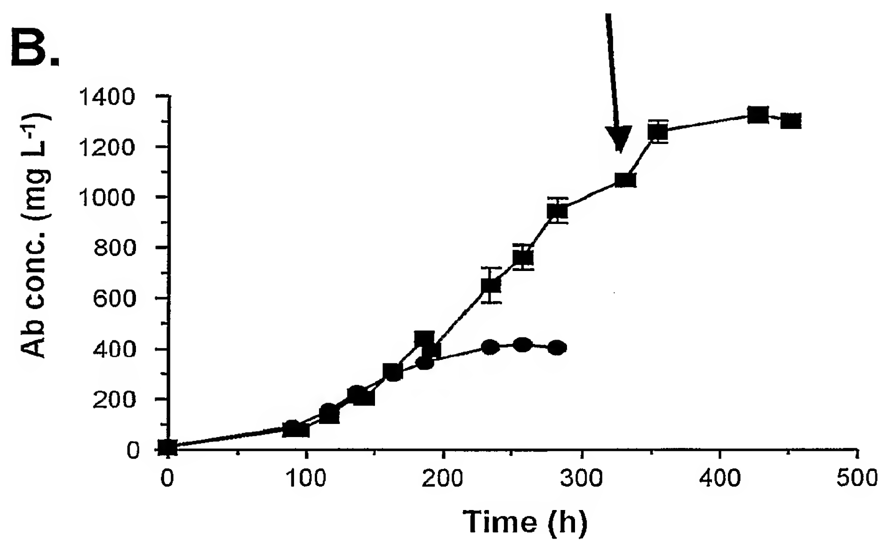

B.
1400
1200
1000
800
600
400
200
0
Ab conc. (mg L^{-1})
0
100
200
300
400
500
Time (h)

Fig. 8

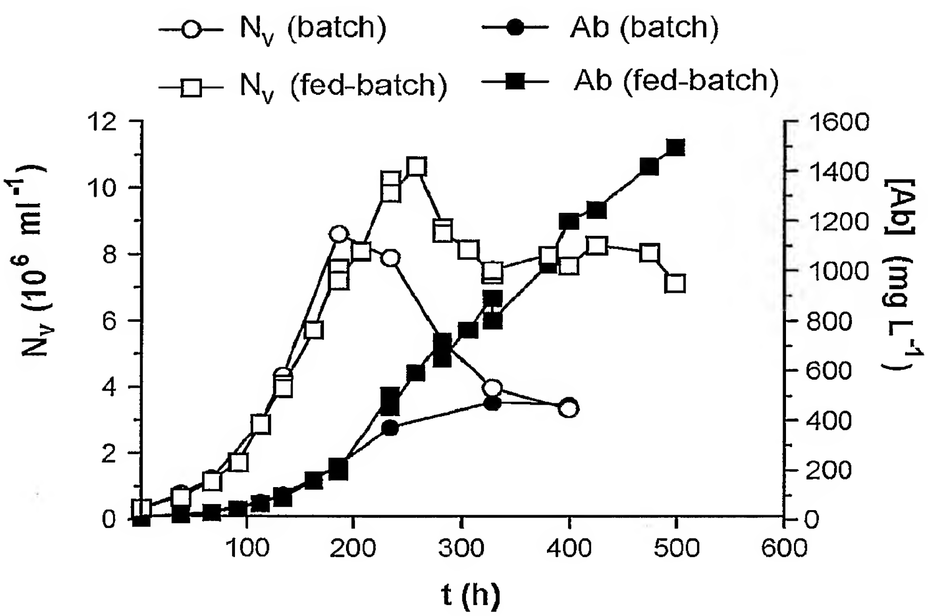

N_V (batch)
Ab (batch)
N_V (fed-batch)
Ab (fed-batch)
N_V (10^6 ml^{-1})
[Ab] (mg L^{-1})
t (h)
0
2
4
6
8
10
12
100
200
300
400
500
600
1600
1400
1200
1000
800
600
400
200

Fig. 9

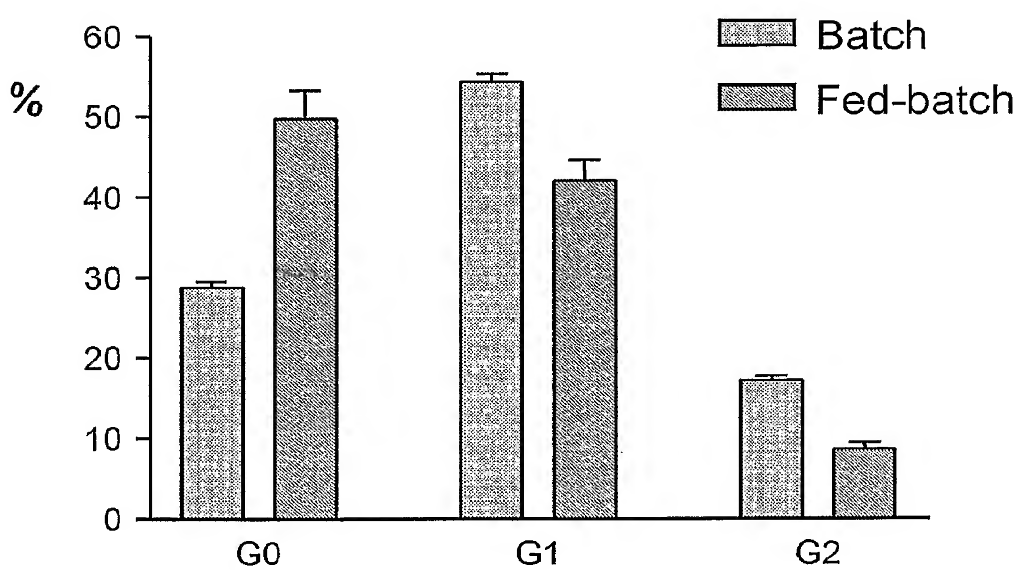
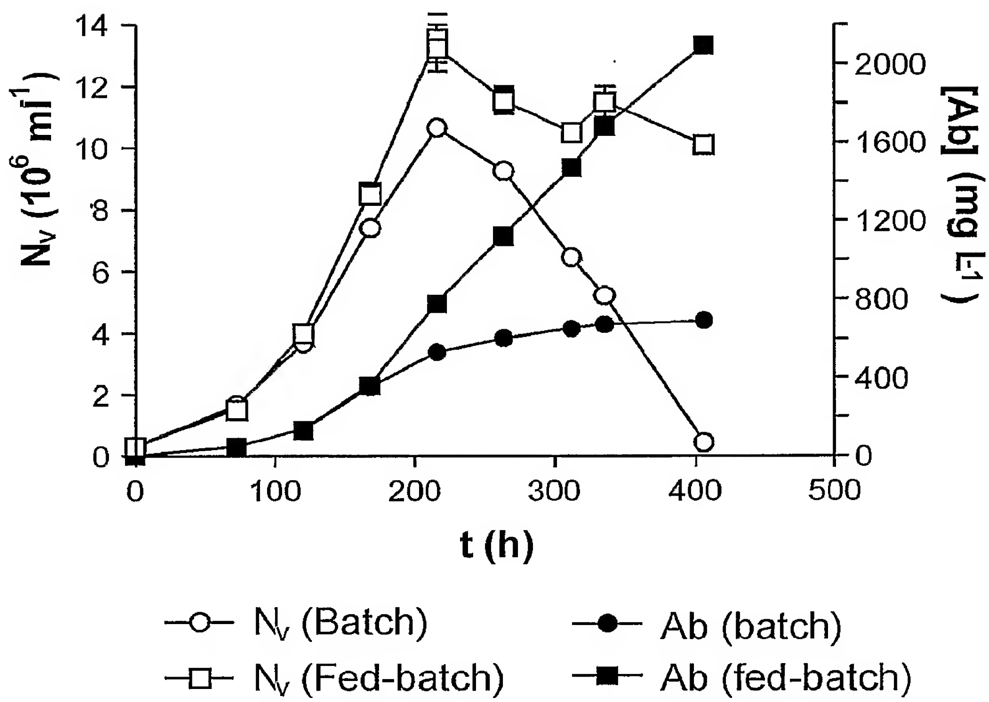


Fig. 10



14
12
10
8
6
4
2
0
N_v (10^6 ml^{-1})
2000
1600
1200
800
400
0
[Ab] (mg L^{-1})
0
100
200
300
400
500
t (h)
N_v (Batch)
N_v (Fed-batch)
Ab (batch)
Ab (fed-batch)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050724

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BOUT B: "PER.C6TM AS PRODUCTION PLATFORM FOR HUMAN MONOCLONAL ANTIBODIES" HUMAN ANTIBODIES, AMSTERDAM, NL, vol. 12, no. 1-2, 8 October 2003 (2003-10-08), page 30, XP009023644 ISSN: 1093-2607 the whole document -----	21-41
X	JONES D ET AL: "High level expression of recombinant IgG in the human cell line PER.C6" BIOTECHNOLOGY PROGRESS, XX, XX, vol. 19, 14 January 2003 (2003-01-14), pages 163-168, XP002256988 ISSN: 8756-7938 figure 5 -----	21-41

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 2004	01/10/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Friedrich, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JONES D H ET AL: "PER.C6 CELL LINE FOR HUMAN ANTIBODY PRODUCTION CRUCELL'S TECHNOLOGY MAINTAINS HUMAN GLYCOSYLATION PATTERNS" GENETIC ENGINEERING NEWS, MARY ANN LIEBERT, NEW YORK, US, vol. 22, no. 10, 15 May 2002 (2002-05-15), page 50,54, XP009018783 ISSN: 1270-6377 the whole document	1-41
A	XIE L ET AL: "SERUM-FREE SUSPENSION CULTIVATION OF PER.C6 CELLS AND RECOMBINANT ADENOVIRUS PRODUCTION UNDER DIFFERENT PH CONDITIONS" BIOTECHNOLOGY AND BIOENGINEERING. INCLUDING: SYMPOSIUM BIOTECHNOLOGY IN ENERGY PRODUCTION AND CONSERVATION, JOHN WILEY & SONS. NEW YORK, US, vol. 80, no. 5, 5 December 2002 (2002-12-05), pages 569-579, XP001155285 ISSN: 0006-3592 figures 2,4	1-41
A	EP 1 108 787 A (CRUCELL HOLLAND B V) 20 June 2001 (2001-06-20) paragraph '0068!	1-41
T	XIE LIANGZHI ET AL: "Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6TM cell culture under sparging conditions." BIOTECHNOLOGY AND BIOENGINEERING, vol. 83, no. 1, 5 July 2003 (2003-07-05), pages 45-52, XP002296365 ISSN: 0006-3592 page 46	1-41
T	PHAM PHUONG LAN ET AL: "Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: Peptone additives improve cell growth and transfection efficiency." BIOTECHNOLOGY AND BIOENGINEERING, vol. 84, no. 3, 5 November 2003 (2003-11-05), pages 332-342, XP002296366 ISSN: 0006-3592 figure 9	1-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/050724

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-20

   Strategy for feeding E1 adenovirus immortalized cells

   ---

2. claims: 21-35

   Culture of E1 adenovirus immortalized cells comprising at least 10 e7 cells.

   ---

3. claims: 36-41

   Culture method for E1 adenovirus immortalized cells comprising a certain culture medium

   ---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/050724

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1108787 A	20-06-2001	EP 1103610 A1	30-05-2001
		EP 1108787 A2	20-06-2001
		AU 775966 B2	19-08-2004
		AU 2557201 A	04-06-2001
		BR 0015846 A	16-07-2002
		CA 2392653 A1	31-05-2001
		CN 1433472 T	30-07-2003
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